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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 9/51, 47/48

A1

(11) International Publication Number: **WO 98/56363**

(43) International Publication Date: 17 December 1998 (17.12.98)

(21) International Application Number: PCT/US98/12126

(22) International Filing Date: 11 June 1998 (11.06.98)

(30) Priority Data:
60/049,496 13 June 1997 (13.06.97) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US 60/049,496 (CIP)
Filed on 13 June 1997 (13.06.97)

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: KNOBBY NANOSPHERES

(57) Abstract

The isolation and purification of adenovirus fiber protein Knob domain is taught, as well as its use as a ligand for intracellular delivery of bioactive agents, such as low molecular drugs, proteins, antisense oligonucleotides, and plasmid DNAs. The conjugation of Knob to the surface of DNA–nanospheres facilitates the binding of nanospheres to cell surfaces and enhances transfection efficiency of DNA–nanospheres.

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KNOBBY NANOSPHERES

TECHNICAL FIELD OF THE INVENTION

This invention is related to improved vehicles for delivering substances to the intracellular milieu.

BACKGROUND OF THE INVENTION

Delivery of therapeutic agents to cells and efficient transfer of the agents into the cells continue to be major obstacles to effective treatments. Thus some agents lose potency by degradation in the body before they reach their desired target cells or tissue. In addition, even if an agent reaches its target tissue or cells, failure to translocate the agent into the cells can prevent the agent from reaching the target molecules, thus preventing effective therapy.

Thus there is a continuing need in the art of pharmacology for vehicles for efficient delivery of drugs to their target molecules.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a solid nanoparticle for delivery of therapeutic agents to target cells and for cell binding.

It is another object of the present invention to provide a method of forming solid nanoparticles for delivery of therapeutic agents to target cells.

It is yet another object of the present invention to provide a method for introducing genes into cells.

These and other objects of the invention are achieved by providing a solid nanoparticle for delivery to target cells. The nanoparticle comprises a polymeric cation and a polyanion, wherein the polyanion consists of nucleic acids, wherein a

polypeptide is attached to the surface of said nanoparticle, wherein the polypeptide comprises the knob domain of adenovirus fiber protein.

According to another embodiment of the invention a method of forming solid nanoparticles for delivery to target cells is provided. The method comprises the steps of:

forming solid nanoparticles by coacervation of a polyanion consisting of nucleic acids and a polymeric cation;

adhering a molecular species to the surface of the nanoparticles wherein the molecular species is selected from the group consisting of a polypeptide comprising the knob domain of adenovirus fiber protein and a linking molecule, wherein if the molecular species is a linking molecule the method further comprises the step of binding a polypeptide comprising the knob domain of adenovirus fiber protein to the linking molecule.

In yet another embodiment of the invention a method for introducing genes into cells is provided. The method comprises the steps of:

incubating (a) cells to be transfected with (b) solid nanoparticles comprising a coacervate of a polymeric cation and a polyanion consisting of nucleic acids, wherein a polypeptide is attached to said nanoparticles' surface, said polypeptide comprising the knob domain of adenovirus fiber protein.

According to yet another aspect of the invention a gene delivery vehicle is provided. A polypeptide is attached to the surface of the gene delivery vehicle. The polypeptide comprises the knob domain of adenovirus fiber protein.

These and other embodiments of the invention which will be apparent to those of skill in the art from a thorough and complete reading of the disclosure, provide the art with methods and reagents for improved delivery of therapeutic agents to target cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Improvement of transfection efficiency of DNA-chitosan nanospheres into 293 cells with Knob conjugated on the surface of the nanospheres. The DNA encoded luciferase. Luciferase activity is shown.

Figure 2. Improvement of transfection efficiency of DNA-chitosan nanospheres into HeLa cells with Knob conjugated on the surface of the nanospheres. The DNA encoded luciferase. Luciferase activity is shown.

DETAILED DESCRIPTION

It is the discovery of the present inventors that the Knob domain of the fiber protein of adenovirus can be conjugated to nanospheres or other gene delivery vehicles and that they enhance the uptake of the gene delivery vehicles by cells. Thus it appears that the Knob domain functions as a specific ligand to a cell surface component, and that the binding of the ligand to the cell surface component effects internalization of the gene delivery vehicles.

Nanospheres are solid particles made by the complex coacervation of a polycation and a polyanion. The polycation can be gelatin, actin, tubulin, cytochromoe C, serum albumin, or histones, or other similar positively charged protein. The polycation can also be a polysaccharide such as chitosan, proteoglycan, methylcellulose, amylose, or starch. The polyanion can be nucleic acids or chondroiton sulfate, for example. Other components such as low molecular weight drugs, proteins, antisense oligonucleotides, and nucleic acids such as plasmid DNAs can also be encapsulated in the nanosphere. Other gene delivery vehicles which may be used include protein-DNA complexes, gold particles, liposomes, and polymeric nanoparticles.

Fiber protein is one of the capsid components of human adenovirus. Fiber protein or a portion of fiber protein, in particular the knob domain, can be covalently attached to a nanosphere. Although not wishing to be bound by any particular theory or mechanism of action, applicants believe that fiber protein

facilitates the internalization of nanospheres by cells, perhaps by a specific interaction with a receptor protein. This internalization permits more drug or therapeutic agent to reach the intracellular target, thus permitting lower doses to be used than without the fiber protein. This provides both a cost savings and a safety benefit.

The surface of nanoparticles or gene delivery vehicles can be easily derivatized for the direct coupling of targeting moieties. For example, carbodiimides can be used as a derivatizing agent. Alternatively, spacers (linking molecules and derivatizing moieties on targeting ligands) such as avidin-biotin can be used to indirectly couple targeting ligands to the nanoparticles. Biotinylated antibodies and/or other biotinylated ligands can be coupled to the avidin-coated nanoparticle surface efficiently because of the high affinity of biotin ($k_a \sim 10^{15}$ M^{-1}) for avidin (Hazuda, et al., 1990, Processing of precursor interleukin 1 beta and inflammatory disease, *J. Biol. Chem.*, **265**:6318-22; Wilchek, et al., 1990, Introduction to avidin-biotin technology, *Methods In Enzymology*, **184**:5-13). Orientation-selective attachment of IgGs can be achieved by biotinylating the antibody at the oligosaccharide groups found on the F_C portion (O'Shannessy, et al., 1984, A novel procedure for labeling immunoglobulins by conjugation to oligosaccharides moieties, *Immunol. Lett.*, **8**:273-277). This design helps to preserve the total number of available binding sites and renders the attached antibodies less immunogenic to F_C receptor-bearing cells such as macrophages. Spacers other than the avidin-biotin bridge can also be used, as are known in the art. For example, Staphylococcal protein A can be coated on the nanoparticles for binding the F_C portions of immunoglobulin molecules to the nanoparticles.

Cross-linking of linking molecules or targeting ligands to the nanoparticle is used to promote the stability of the nanoparticle as well as to covalently affix the linking molecule or targeting ligand to the nanoparticle. The degree of cross-

linking directly affects the rate of nucleic acids release from the microspheres. Cross-linking can be accomplished using glutaraldehyde, carbodiimides such as EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, DCC (N,N'-dicyclohexylcarbodiimide), carboxyls (peptide bond) linkage, DSS (Disuccinimidyl suberate), SPDP (N-succinimidyl 3-[2-pyridyldithio]propionate bis (sulfosuccinimidyl) suberate), dimethylsuberimidate, etc.

The nanoparticles of the present invention have good loading properties. Typically, following the method of the present invention, nanoparticles having at least 5% (w/w) nucleic acids can be achieved. Preferably the loading is greater than 10 or 15% nucleic acids. Often nanoparticles of greater than 20 or 30%, but less than 40 or 50% nucleic acids can be achieved. Typically encapsulation efficiencies of nucleic acids into nanoparticles of greater than 95% can be achieved.

The method of the present invention involves the coacervation of polymeric cations and nucleic acids. Because this process depends on the interaction of the positively charged polymeric cations and the negatively charged nucleic acids it can be considered as a complex coacervation process. However, sodium sulfate (or ethanol) induces the coacervation reaction by inducing a phase transition, and therefore it could also be considered as a simple coacervation reaction. Nucleic acids are present in the coacervation mixture at a concentration of between 1 ng/ml to 500 μg/ml. Desirably the nucleic acids are at least about 2-3 kb in length. Sodium sulfate is present at between 7 and 43 mM. Gelatin or other polymeric cation is present at between about 2 and 7% in the coacervation mixture.

Unlike viral vectors, which cannot deliver genes larger than 10 kb, the nanoparticle delivery system of the present invention does not have such size limitations. Nucleic acid molecules of greater than about 2 kb can be used, and nucleic acid molecules even greater than 10 kb may be used. Typically nucleic

acids in the range of 2 to 10 kb, 5 to 15 kb, and even 10-50 kb can be encapsulated.

In general, the range of possible targets is dependent on the route of injection, *e.g.*, intravenous or intraarterial, subcutaneous, intra-peritoneal, intrathecal, etc. For systemic injections, the specificity of this delivery system is affected by the accessibility of the target to blood borne nanoparticles, which in turn, is affected by the size range of the particles. Size of the particles is affected by temperature, component concentration, and pH in the coacervation mixture. The particles can also be size-fractionated, *e.g.*, by sucrose gradient ultracentrifugation. Suitable sizes of nanoparticles are less than 3 μm, preferably less than 2 μm, 1 μm, and even as low as 0.1 μm. Particles with size less than 150 nanometers can access the interstitial space by traversing through the fenestrations that line most blood vessels walls. Under such circumstances, the range of cells that can be targeted is extensive. An abbreviated list of cells that can be targeted includes the parenchymal cells of the liver sinusoids, the fibroblasts of the connective tissues, the cells in the Islets of Langerhans in the pancreas, the cardiac myocytes, the Chief and parietal cells of the intestine, osteocytes and chondrocytes in the bone, keratinocytes, nerve cells of the peripheral nervous system, epithelial cells of the kidney and lung, Sertoli cells of the testis, etc. The targets for particles with sizes greater than 0.2 microns will be confined largely to the vascular compartment. Here, the targetable cell types include erythrocytes, leukocytes (i.e. monocytes, macrophages, B and T lymphocytes, neutrophils, natural killer cells, progenitor cells, mast cells, eosinophils), platelets, and endothelial cells.

For subcutaneous injections, the targetable cells include all cells that resides in the connective tissue (*e.g.*, fibroblasts, mast cells, etc.), Langerhans cells, keratinocytes, and muscle cells. For intrathecal injections, the targetable cells include neurons, glial cells, astrocytes, and blood-brain barrier endothelial cells.

For intraperitoneal injection, the targetable cells include the macrophages and neutrophil.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLE 1

Expression and Purification of the Knob Domain of the Adenovirus Type 5 Fiber Protein

The knob domain was cloned by PCR amplification using cloned Ad5 plasmid DNA (pJM17, McGrory, et al., 1988) as the template and specific oligonucleotides designed to facilitate the insertion of the PCR product into the bacterial expression vector pET15b (Novagen). The sequences of the two primers used were CTCGAGGGTGCCATTACAGTAGGAAACAAAAATAATGATAAG (5' oligonucleotide) and GGATCCTTATTCTTGGGCAATGTATGAAAAAGTGTAAGAGG (3' oligonucleotide), which are partially complementary to specific Ad5 fiber sequences (Chroboczek and Jacrot, 1987). The PCR product of approximately 600 bp was purified by the PCR purification kit (Qiagen), and digested by BamHI and XhoI, then directionally ligated into BamHI-XhoI-digested pET15b. *E. coli* BL21(DE3) cells used as the host strain. A clone containing the appropriate recombinant plasmid was identified by restriction enzyme digestion. The expression of the knob was induced by 0.1 mM IPTG in LB medium and characterized by Western Blot.

EXAMPLE 2

Isolation and purification of the knob domain of adenovirus fiber protein

Purification of the recombinant knob domain from an *E. coli* cell lysate was accomplished using ProBond™ resin (Invitrogen), which has a high affinity for six tandem histidine residues. The pET15b vector used to clone the know domain carries the sequence encoding polyhistidine residues followed by the BamHI-XhoI cloning site, therefore the recombinant knob protein contains a His-Tag in the N-terminus. Cells from 100 ml of culture were spun down and resuspended in 18 ml of Native Binding Buffer (20 mM phosphate, 500 mM NaCl, pH 7.8). One ml of 1% Triton X-100™ was added to achieve good solubilization. The cells were disrupted by sonication in short bursts (20 sec/burst, total 80 seconds), and the cell debris and DNA were precipitated at 10,000 x g for 10 minutes. The pre-equilibrated resin in the column was resuspended with four 5 ml lysate aliquots and gently rocked for 10 minutes to allow for binding of the polyhistidine-containing protein. The resin was then settled by centrifugation, and the supernatant was aspirated. The column was washed three times with 4 ml of Native Binding Buffer, twice with 4 ml Native Wash Buffer (20 mM phosphate, 500 mM NaCl, pH 6.0), then several times with 4 ml Imidazole Buffer (200 mM imidazole, 20 mM phosphate, 500 mM NaCl, pH 6.0), until the OD_{280} was less than 0.01. The recombinant Knob was eluted by 50 mM EDTA, dialyzed and concentrated, yielding approximate one mg.

EXAMPLE 3

Conjugation of Knob to DNA-nanospheres

Free sulfhydryl groups were introduced into Knob protein by 2-iminothiolane method [Hermanson, 1996] with the following modification: a cold solution of 5 mg of knob protein in 2.5 mL of 0.1 M sodium phosphate buffer (pH7.6, 5mM EDTA) was mixed with 120 mL of 2-iminothiolane solution (5 mg/mL in water). The mixture was kept on ice for 2 hours, and pooled over a

PD-10 column. The column was eluted with phosphate buffered saline (PBS) at room temperature, the peak fractions were collected and the concentration of Knob derivative, hereafter called Knob-SH, was determined by absorbance at 280nm.

For Knob conjugation, DNA-chitosan nanospheres were mixed with 5mL of DSS solution (10 mM in DMSO), 10 mL of SPDP solution (10 mM in DMSO) and 100 mL of PBS buffer under vertexing. The mixture was stirred at room temperature for 30 min before 50 mL of 1 M glycine was added to quench the reaction, followed by addition of 250 mg of Knob-SH. After reaction for 60 minutes, the mixture was subjected to ultracentrifugation to harvest the nanospheres.

EXAMPLE 4

***In vitro* Transfection**

Nanospheres containing 1 mg of DNA were incubated with 1.0-5.0 x 105 cells in each well (pre-plated in 12 well plate) at 37° C and 5% CO_2 in DMEM containing 1% fetal bovine serum (FBS), 2 mM L-glutamine, 50 units/mL penicillin, 50 mg/mL streptomycin, and 10 mg/mL gentamycin for 4 hours, followed by changing the medium to fresh complete medium (DMEM containing 10% FBS). Cells were cultured for 3 days before assay. Transfection using Lipofectamine (BRL, Gaithersburg, MD, liposome method) was used as control. Luciferase gene expression levels were measured by assaying Luciferase activity in permeabilized cell extracts (Promega, Madison, WI). The light units (LU) were normalized to protein concentration in the cell extracts measured by the BCA method.

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G. T. Hermanson, Bioconjugate Techniques, Academic Press, Inc. (San Diego, California) 1996.

McGrory, W. J., D. S. Bautista, and F. L. Graham. 1988. A simple technique for the rescue of early region 1 mutations into infectious human adenovirus type 5. J. Virol. 163:614-617.

Philipson, L., K. Lonberg-Holm, and U. Pettersson. 1968. Virus-receptor interaction in an adenovirus system. J. Virol. 2:1064-1075.

CLAIMS

1. A solid nanoparticle for delivery to target cells, said nanoparticle comprising a polymeric cation and a polyanion, wherein the polyanion consists nucleic acids, wherein a polypeptide is attached to the surface of said nanoparticle, wherein the polypeptide comprises the knob domain of adenovirus fiber protein.

2. The nanoparticle of claim 1 wherein the polypeptide is attached to said nanoparticle via a linking molecule.

3. The nanoparticle of claim 1 wherein the polypeptide is directly attached to said nanoparticle.

4. The nanoparticle of claim 2 wherein the linking molecule is covalently attached to said nanoparticle by means of glutaraldehyde cross-linking.

5. The nanoparticle of claim 2 wherein the linking molecule is covalently attached to said nanoparticle by means of SPDP cross-linking.

6. The nanoparticle of claim 2 wherein said linking molecule is avidin.

7. The nanoparticle of claim 2 wherein said linking molecule is polyethylene glycol.

8. The nanoparticle of claim 2 wherein the polypeptide is covalently coupled to the linking molecule.

9. The nanoparticle of claim 1 wherein said polymeric cation is gelatin.

10. The nanoparticle of claim 1 wherein said polymeric cation is chitosan.

11. The nanoparticle of claim 1 which comprises from 5% to 30% (w/w) nucleic acids.

12. The nanoparticle of claim 1 wherein said nanoparticle comprises 20% to 30% (w/w) nucleic acids.

13. The nanoparticle of claim 6 wherein a biotinylated knob domain is coupled to the linking molecule.

14. The nanoparticle of claim 1 wherein said nucleic acids comprises a gene of 10 to 50 kb.

15. A method of forming solid nanoparticles for delivery to target cells, the method comprising the steps of:

forming solid nanoparticles by coacervation of a polyanion consisting of nucleic acids and a polymeric cation;

adhering a molecular species to the surface of the nanoparticles wherein the molecular species is selected from the group consisting of a polypeptide comprising the knob domain of adenovirus fiber protein and a linking molecule, wherein if the molecular species is a linking molecule the method further comprising the step of binding a polypeptide comprising the knob domain of adenovirus fiber protein to the linking molecule.

16. The method of claim 15 further comprising the steps:

conjugating the molecular species to the nanoparticles.

17. The method of claim 15 wherein the coacervation is performed in the presence of sodium sulfate.

18. The method of claim 15 wherein the polymeric cation is gelatin.

19. The method of claim 15 wherein the linking molecule is avidin.

20. The method of claim 15 wherein the linking molecule is polyethyleneglycol.

21. The method of claim 15 wherein the polypeptide is modified with a moiety which binds to the linking molecule.

22. The method of claim 18 wherein the gelatin is present at a concentration of about 2-7% in the step of coacervation.

23. The method of claim 15 wherein the nucleic acids are present in a concentration of 1 ng/ml to 500 μg/ml in the step of coacervation.

24. The method of claim 17 wherein the concentration of sodium sulfate is between about 7 and 43 mM in the step of coacervation.

25. A method for introducing genes into cells comprising the steps of:

incubating (a) cells to be transfected with (b) solid nanoparticles, said nanoparticles comprising a coacervate of a polymeric cation and a polyanion consisting of nucleic acids, wherein a polypeptide is attached to said nanoparticles' surface, said polypeptide comprising the knob domain of adenovirus fiber protein.

26. The method of claim 25 wherein the polypeptide is attached to said nanoparticle's surface by means of a linking molecule.

27. The method of claim 26 wherein the linking molecule is avidin.

28. The method of claim 25 wherein the polymeric cation is gelatin.

29. The method of claim 25 wherein the nucleic acid is DNA.

30. The method of claim 25 wherein the nucleic acid is RNA.

31. The nanoparticle of claim 1 wherein said nucleic acids comprise a gene of 2 to 10 kb.

32. A gene delivery vehicle, wherein a polypeptide is attached to the surface of said gene delivery vehicle, wherein the polypeptide comprises the knob domain of adenovirus fiber protein.

33. The gene delivery vehicle of claim 32 wherein the polypeptide is attached to said vehicle via a linking molecule.

34. The gene delivery vehicle of claim 32 wherein the polypeptide is directly attached to said gene delivery vehicle.

35. The gene delivery vehicle of claim 33 wherein the linking molecule is covalently attached to said gene delivery vehicle by means of glutaraldehyde cross-linking.

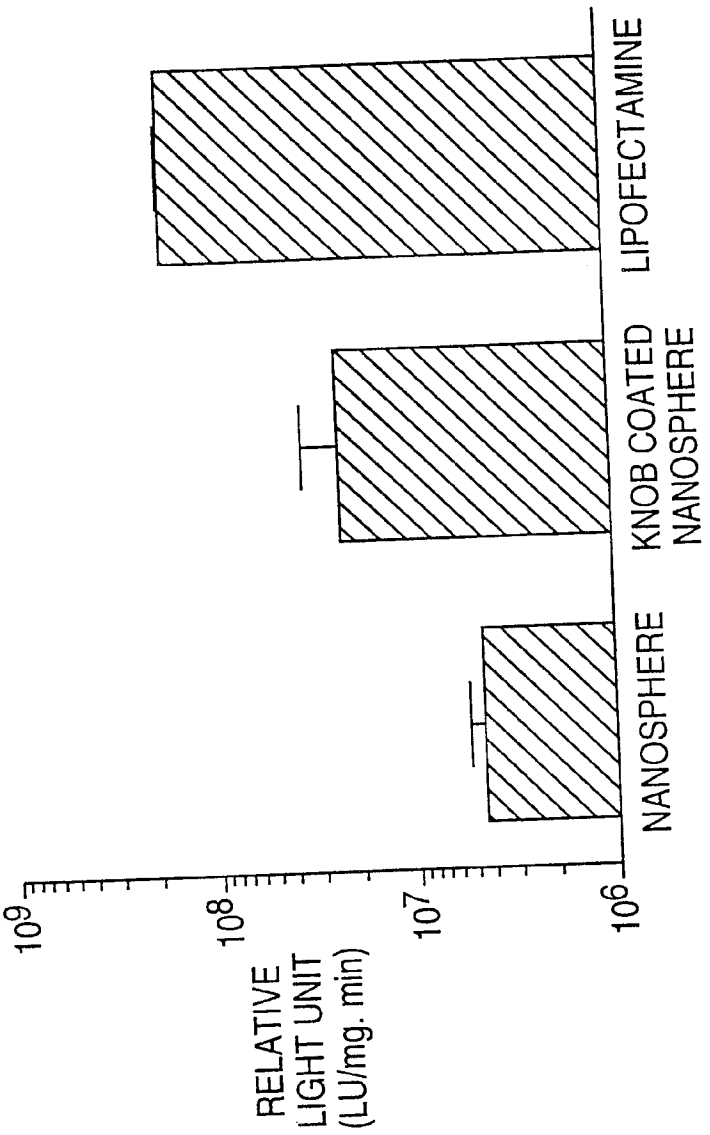
36. The gene delivery vehicle of claim 33 wherein said linking molecule is avidin.

37. The gene delivery vehicle of claim 32 which is a liposome.

38. The gene delivery vehicle of claim 32 which is a protein-DNA complex.

39. The gene delivery vehicle of claim 32 which is a gold particle.

FIG. 1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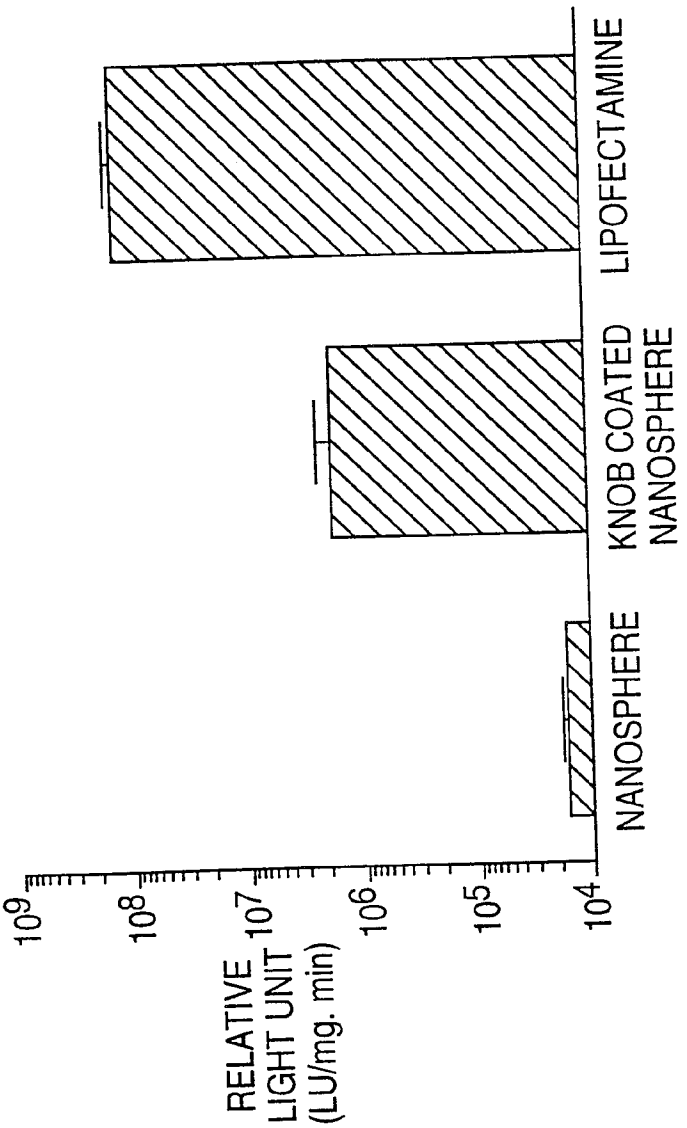
10^8
10^7
10^6
NANOSPHERE
KNOB COATED NANOSPHERE
LIPOFECTAMINE

FIG. 2


RELATIVE LIGHT UNIT (LU/mg. min)
10^9
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10^5
10^4
NANOSPHERE
KNOB COATED NANOSPHERE
LIPOFECTAMINE

INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/US 98/12126

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K9/51 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	WO 98 01162 A (UNIV JOHNS HOPKINS) 15 January 1998 see claims	1-39
P,A	MAO H.-Q. ET AL: "DNA-chitosan nanospheres: Derivatization and storage stability." PROCEEDINGS OF THE CONTROLLED RELEASE SOCIETY, (1997) -/24 (671-672). REFS: 4 ISSN: 1022-0178 CODEN: 58GMAH, XP002079941 United States see page 671, column 1, paragraph 1 - paragraph 2	1-39

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
9 October 1998	20/10/1998

Name and mailing address of the ISA	Authorized officer
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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/US 98/12126

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,A	ROY, KRISHNENDU ET AL: "DNA - chitosan nanospheres: transfection efficiency and cellular uptake" PROC. INT. SYMP. CONTROLLED RELEASE BIOACT. MATER. (1997), 24TH, 673-674 CODEN: PCRMEY;ISSN: 1022-0178,1997, XP002079942 see figure 3 ---	1-39
P,X	WO 97 28817 A (CHENG PI WAN) 14 August 1997 see claims 1,4,11,19-21 ---	32-39
X	C.K. GOLDMAN ET AL.: "TARGETED GENE DELIVERY TO KAPOSI'S SARCOMA CELLS via THE FIBROBLAST GROWTH FACTOR RECEPTOR." CANCER RESEARCH, vol. 57, no. 8, 15 April 1997, pages 1447-1451, XP002079943 MD US	32-39
Y	see abstract ---	32-39
Y	DOUGLAS J T ET AL: "Strategies to accomplish targeted gene delivery to muscle cells employing tropism-modified adenoviral vectors." NEUROMUSCULAR DISORDERS, (1997 JUL) 7 (5) 284-98. REF: 98 JOURNAL CODE: BJS. ISSN: 0960-8966., XP002079944 ENGLAND: United Kingdom see page 288, column 1, paragraph 2; figures 2,3,12	32-39
X	see page 296, column 1, paragraph 3 ---	32
X	ROGERS B E ET AL: "USE OF A NOVEL CROSS-LINKING METHOD TO MODIFY ADENOVIRUS TROPISM" GENE THERAPY, vol. 4, no. 12, December 1997, pages 1387-1392, XP002069405 see abstract; figure 2 ---	32-39
X	DATABASE MEDLINE US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US DOUGLAS J T ET AL: "Targeted gene delivery by tropism-modified adenoviral vectors." XP002079945 see abstract & NATURE BIOTECHNOLOGY, (1996 NOV) 14 (11) 1574-8. JOURNAL CODE: CQ3. ISSN: 1087-0156., United States -----	32-39

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/ 12126

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 25-30
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 25-30 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internal Application No
PCT/US 98/12126

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9801162 A	15-01-1998	AU 3739697 A	02-02-1998
WO 9728817 A	14-08-1997	AU 2112697 A	28-08-1997